ANIMAL CELLS AND MICROBIOLOGY[1]

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Of the many distinguished presidential addresses in the archives of our Society, some represent important contributions to the history of microbiology; others have surveyed the contemporary scientific scene with humor and insight; and a few have ventured to foretell the shape of things to come. In any case, precedent almost requires that on this occasion your president deal with the general rather than the particular, and with the controversial rather than the factual. However, I must decline this opportunity to philosophize. I choose to defer the thoughtful remembrance of things past; and it would be a venturesome man indeed who in today's expanding universe of science would dare to extrapolate from the past into the future.

As to the present, there is to be sure much in the world about us that deserves and invites comment. A large segment of our population has recently discovered that science is important. Unfortunately, that realization has been brought home by immediate and urgent practical needs. Even now, I am not convinced that the necessity for basic research unrelated to an immediate objective is understood or accepted by some of those who most insistently bespeak its applications. The importance of this pursuit of knowledge for knowledge's own sake is not a rationalization by unworldly scientists attempting to escape reality. It is a truism that the esoteric and apparently useless information of today unpredictably becomes the essential keypiece in the practical application of tomorrow, or next year, or 20 years hence. But even if this were as self-evident to all as it is to most scientists, the problems remain of how to achieve a proper balance between applied research and the mainstream of scientific progress, and of how to provide the proper educational, economic, and political climate for the most fruitful development of science and scientists. All of us have opinions and perhaps convictions on these complex and multifaceted problems. I have, however, been too immersed in my own day-by-day preoccupations to be able to generalize with confidence, or to feel that my views are so obviously right that I can or should use this office and this occasion as a forum from which to propagandize; and besides, this would not be the right audience. Like the fictional juggler of Notre Dame I am therefore constrained to render my presidential homage to the Society in terms of my work experience; but I take comfort in the fact that many others before me have similarly chosen to evade their presidential responsibility by offering their experimental data, with more or less justifiable pride and with obvious affection.

I

For several years my associates[2] and I have been studying the nutritional requirements and metabolic activity of human and animal cells in cultures. Surprisingly, every cell strain so far examined, no matter what the species or organ from which it was derived, and whether normal or malignant in origin, has proved to require essentially the same amino acids, vitamins, and salts, and at approximately the same concentrations. This metabolic uniformity, and the difficulties so far encountered in the production or identification of biochemical mutants, contrast sharply with the extraordinary diversity and variability of microorganisms. As shown in table 1, a total of only 28 defined growth factors (13 amino acids, 8 vitamins, 6 ions, and glucose) supplemented with serum protein, have so far proved adequate for the cultivation of all the human cell lines examined; and a total of 32 factors provide for the sustained propagation of every mammalian cell strain so far isolated. On the omission of a single one of these essential factors from the complete medium, growth

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TABLE 1

Minimum nutritional requirements for human cell cultures

Amino Acids or Precursors		Carbohydrate	Vitamins or Cofactors*	
Arginine		Glucose	Choline	
Histidine		Fructose	Folic	Folinic
Isoleucine		Galactose	Nicotinamide	DPN
Leucine		Mannose		TPN
Lysine		Ribose (+ pyruvate)	Pantothenate	Co-A
Methionine			Pyridoxal	
Phenylalanine			Riboflavin	FAM, FAD
Threonine			Thiamin	Co-Carb
Tryptophan			Inositol	
Tyrosine				
Valine				
Glutamine	Glutamic	Ions	Protein	
	Aspartic	Na^+	Serum protein	
Cyst(e)ine	Homocystine	K^+		
	Cystathionine	Cl^-		
	Glutathione	Ca^{++}		
	Thioglycollate	Mg^{++}		
	S^-, $S_2O_3^-$, $S_2O_4^-$, SO_3^-	$H_2PO_4^-$		

* DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FAM, flavin adenine mononucleotide; FAD, flavin adenine dinucleotide; Co-A, coenzyme A; Co-carb, cocarboxylase.

ceases, typical degenerative changes develop, and the cells die.

The responses to the individual growth factors are as precise as those of a microbiological assay; and as with bacteria, amino acids in excess are often inhibitory. In the case of bacteria, this unfavorable effect of amino acid imbalance on growth has in some instances been shown to be related to amino acid transport into the cell, and can often be rectified by appropriate changes in the concentrations of the other amino acids. It remains to be seen whether this is true also of the animal cells.

The nutritional requirements of these cultured cells differ importantly from those of the whole animal. Although man requires only 8 amino acids for nitrogen balance, every cell strain so far examined has required at least 13 for survival and growth. Arginine, histidine, glutamine, cystine, and tyrosine are essential amino acids in cell cultures, but not in man. This discrepancy is not the result of their prolonged cultivation; for when monkey kidney cells were tested in primary culture, within 24 hours after their isolation from the animal, they were found to require the same 13 amino acids as cells which had been serially propagated for years.

One possible explanation is that the minimal medium in which these experiments were carried out, and which included only the demonstrably essential growth factors, may have lacked precursors or cofactors required for the synthesis of these particular amino acids. This has been shown to be the case for cyst(e)ine. In the whole animal, cyst(e)ine can be formed from methionine by way of homocysteine and cystathionine; and in these cell cultures, a block in the demethylation of methionine is apparently the growth-limiting reaction. However, human cell strains can utilize homocysteine for cysteine synthesis, and can cleave cystathionine to cystine in amounts sufficient for growth. Further, we have recently learned that, like *Escherichia coli* and *Salmonella* sp., most of the human cell strains can effect the synthesis of cystine from reduced inorganic sulfur compounds, but not from sulfate. The origin of the carbon skeleton is not yet clear. Finally, and surprisingly, many of the cell lines studied can utilize D-cyst(e)ine or D-homocyst(e)ine as effectively as the L-isomers. Labeling experiments

have shown that the D-isomers are desulfurated, and that the sulfide so formed is used for cystine biosynthesis. Whether in addition the cell can effect the racemization uniquely of these D-amino acids, remains to be determined.

It is conceivable that arginine, glutamine, histidine, and tyrosine are similarly made in trace amounts from simple precursors, or from other essential amino acids, at a rate sufficient for short-term nitrogen balance *in vivo*, but inadequate for the rapid growth characteristic of these cell cultures. Glutamine and tyrosine are cases in point. Glucose is used by these cells to a limited degree for the biosynthesis of glutamic acid; and glutamic acid in turn can to a limited degree be used for glutamine synthesis. However, the amount of glutamine so produced does not normally suffice for growth, or even for survival. One is at once reminded of the similar findings by Snell and others in lactobacilli. In both the microorganism and the animal cell, this glutamine requirement is satisfied by high concentrations of glutamic acid; and quite recently DeMars in our laboratory has shown that at those high concentrations of glutamic acid, cultured animal cells develop increased amounts of glutamine synthase. Having formed the enzyme, they can then make adequate amounts of glutamine and grow even at low levels of glutamic acid. This may be the first reported example of enzyme induction in animal cell cultures; and we may hope for the demonstration of many others.

Another example of a limited biosynthesis inadequate for growth is provided by tyrosine. None of the serially propagated cell strains so far studied has been able to effect the hydroxylation of phenylalanine to tyrosine. Moreover, the metabolic pathways by which bacteria can make aromatic amino acids, so elegantly elaborated by Davis in *E. coli* mutants, is apparently not operative in the mammalian cell cultures so far studied. Phenylalanine, tyrosine, and tryptophan are all nutritionally essential for optimal growth. One culture, a variant strain of the HeLa cell, was, however, able to hydroxylate phenylalanine to tyrosine. Despite this biosynthetic capacity, this variant strain deteriorated and died in a tyrosine-free medium.

It is worth emphasizing that, as has so often been observed in bacteria, the biosynthesis of glutamine from glutamic acid, and the formation of tyrosine from phenylalanine, were both product-inhibited. The addition of tyrosine to the medium completely prevented its formation from phenylalanine; and DeMars has shown that the presence of glutamine similarly inhibits the formation of glutamine synthase.

Not even trace formation of arginine and histidine has yet been demonstrated in animal cell cultures. One possibility which deserves further exploration is that the requirement for arginine, histidine, and tyrosine, and the almost complete block in the biosynthesis of glutamine are not peculiar to these cultures. Even *in vivo*, most cells may be unable to synthesize these amino acids. In the whole animal they may perhaps be produced by a single organ such as the liver, which could supply the rest of the body with these specific amino acids; and in feeding experiments, the participation of the intestinal flora can of course not be excluded.

The metabolic activity of these animal cells with respect to the nutritionally essential amino acids so far studied differs from that of most bacteria in that, with only one exception, they are not metabolized to any important degree, but are simply used for incorporation into protein. The exception is glutamine. In a minimal medium which embodies only those growth factors demonstrably essential for survival and growth, glutamine serves as the primary source for glutamic acid, aspartic acid, asparagine, and proline. In that medium the cell is making all of its nucleic acid from glucose and amino acids. Forty per cent of the pyrimidine carbon derives from glutamine, and more than 50 per cent of the total purine and pyrimidine nitrogen, including the amino groups of guanine and cytosine, derive specifically from glutamine amide nitrogen. Unlike the case of bacteria, free ammonia is not used by these cells to any significant degree, whether for the biosynthesis of nucleic acids or amino acids.

In their amino acid requirements, these cultured cells have proved to be more auxotrophic than the whole animal, requiring at least 4 more amino acids. Their vitamin requirements, however, are apparently considerably less than those of the intact animal. Only 8 vitamins have so far been identified as demonstrably essential for the growth of animal cells in culture: choline, folic acid, inositol, nicotinamide, pantothenate, pyro-

doxal, riboflavin, and thiamin. Inositol, which was the first of the vitamins to be discovered, is required by yeasts and certain fungi, but its significance in mammalian physiology had been questioned by many workers. In human and animal cell cultures, it proved to be essential for survival and growth of 21 out of 22 cell lines tested. The exception was a mouse fibroblast which not only grew in an inositol-free medium, but, in preliminary experiments, apparently released inositol into the medium in amounts sufficient to feed other cultures requiring inositol If confirmed, this will be the first example of syntrophism in dispersed mammalian cell cultures.

Surprisingly, neither the fat-soluble vitamins, ascorbic acid, B_{12}, nor biotin appear in the list of the essential vitamins of table 1. Almost certainly, some of these are essential for growth, but are present as trace contaminants either in the other defined components of the medium, or bound to the serum protein, from which they could be slowly released in amounts sufficient for growth. It is also probable that a certain number of vitamins are not required for growth, but are essential for those specialized cellular functions which are so conspicuously absent in these serially propagated cultures.

Puck and his associates have shown that single animal cells may be planted on a glass surface to yield clones, analogous to the single cell colonies grown by the bacteriologist. On the basis of results obtained in microorganisms, we should perhaps not have been surprised to find that the minimal growth medium of 28 defined growth factors and serum protein which had regularly sufficed for the growth of large inocula, did not permit the growth of single cells. The capacity of the cells to grow was, however, completely restored by the addition of a full complement of the ordinarily nonessential amino acids; and, in most of the experiments, serine alone was as active as the complete mixture. In partial explanation, when extremely small numbers of cells are suspended in a medium which lacks the ordinarily nonessential amino acids, the equilibration between the medium and the cells results in the loss of amino acids from the cell pool at a rate which may exceed its biosynthetic capacity.

This amino acid pool has proved of critical importance in the production of poliovirus by the HeLa cell. In that system, viral protein is apparently synthesized from the amino acids of the pool, and does not derive from breakdown products of cellular protein. The ability of the cells to form essentially normal amounts of virus in a medium containing only glucose and glutamine is referable to the presence of a full complement of amino acids in the pool. (It is of interest in this connection that the amounts of glucose and glutamine required for maximal elaboration of virus are precisely the same as those required for the growth of the cell.) When some of those amino acids are sufficiently depleted, as for example by vitamin B_6 starvation, or by dilution of the cell suspension in an appropriately deficient medium, viral synthesis is greatly decreased, and is restored to normal levels by the addition of the missing amino acids to the medium.

II

It is obvious that only a beginning has been made in the study of metabolism of animal cells in culture. The degree to which these cultures will provide an approach to the problem of embryonic differentiation and of cellular function remains to be determined, for to date no specialized functions have been retained by serially propagated dispersed cell cultures. One may nevertheless anticipate that this will eventually be accomplished; and at that point the study of these cell cultures becomes the study of animal physiology at the cellular level.

The question may perhaps be asked as to what bearing all this has upon microbiology. I believe that these animal cells are not only a valid, but indeed a vital concern of the microbiologist. The techniques which have been used are those which have been developed and so fruitfully exploited over the past few decades by microbiologists. Whether we are discussing nutritional requirements, metabolic pathways, cloning, mutations, feedback mechanisms and product-inhibited reactions, enzyme induction, auxotrophy, or syntrophism: at each step we fall back upon the prototype experiments of the microbiologist. But it is not only the techniques which are similar, but also the findings. Nowhere is the classic unity of biochemical pathways more conspicuously evidenced than here. The parallelism in the amino acid and vitamin requirements of animal cells and lactobacilli is extraordinary; and the pathways by which these

TABLE 2

Nutritional and metabolic differences in cell cultures

Cell Strain	Unusual Nutritional Requirement	Unusual Metabolic Activity	Reference
Walker carcinosarcoma (256)	Asparagine		McCoy, Maxwell, and Neumann
Rabbit fibroblast (RM3)	Serine		Haff and Swim
Monkey kidney cells in primary culture	Glycine		Rappaport and Melnick; Eagle, Levy, and Freeman
		Aspartic, Glutamic → Glutamine	Eagle, Freeman, and Levy
Mouse fibroblast (strain "L")		Biosynthesis of inositol in amounts sufficient for growth	Eagle, Agranoff, and Snell
HeLa "variant"		Phenylalanine → tyrosine	Eagle, Piez, and Fleischman
HeLa clones	Varying concentrations of serum protein		Puck and Fisher
Small populations of human cells	"Nonessential" amino acids, notably serine, required for cloning		Lockart and Eagle
	"Nonessential" amino acids required for poliovirus synthesis		Darnell and Eagle

nutrilities are metabolized to yield the cellular macromolecules will probably prove no less similar. Several points of difference are, however, emerging and deserve emphasis.

One is the metabolic inactivity of ammonia in cell cultures, and the central role of glutamine in amino acid metabolism, protein synthesis, and in particular, nucleic acid synthesis. A second point of difference relates to the stability of the formed proteins, *i.e.*, protein turnover. In bacteria it has been shown in several classic experiments that proteins are extremely stable, and that their amino acid residues are not used by the growing cell for the biosynthesis of either new proteins or nucleic acids. In growing yeast also, Halvorson has shown that there is only negligible protein turnover. In resting yeast cells, however, he has demonstrated a relatively rapid turnover amounting to 0.66 per cent per hr; and recent experiments in several laboratories indicate that there is similar protein turnover in resting bacteria. Relatively speaking, the proteins of mammalian cells are far less stable than those of either bacteria or yeast. In appropriately deficient media, in the absence of net protein synthesis, labeled amino acids are incorporated from the medium into cell protein at the rate of 1 per cent per hr. Compared to a maximum growth rate in these cell cultures of only 3 to 4 per cent per hr, this is an extraordinarily rapid rate of incorporation. Further, unlike bacteria and yeast, a similar process of protein renewal is observed in growing cultures, and at essentially the same rate. It remains to be seen whether this is true protein turnover, in the sense of total degradation of the protein to its component amino acids, followed by resynthesis; or whether instead this is an exchange reaction of the type suggested for bacteria by Gale.

Finally, the metabolic uniformity of animal

cells in culture contrasts sharply with the extraordinary metabolic diversity of bacteria. True, a number of minor nutritional and metabolic differences have been noted between various cell lines (*cf.* table 2); but these differences are minor in relation to their broad similarity. It must be admitted that only a small number of animal tissues have so far been cultivated, and that the type of cell which grew out cannot usually be characterized with certainty. Is it possible that the animal cells so far cultured have been selected from a heterogenous population by that very capacity to grow *in vitro*, and that their metabolic uniformity merely reflects this unintentional and unwanted selection? Or do most animal cells indeed have the same basic requirements for growth? Until proved otherwise, I incline to the latter view. In terms of the nutrilites required, and the pathways of their utilization, cultured human cells resemble lactobacilli. Through millennia of evolution from unicellular organisms to the higher animals, and despite the increasingly complex organization of cells into structures, and their functional specialization, there has been no basic alteration in the biochemical processes involved in the synthesis of macromolecules and in cellular growth. By the same token, when we learn how to maintain subtle specialized functions in cultured cells, we may confidently look forward to the demonstration of correspondingly subtle differences in their metabolic potentialities.

The biochemical uniformity in the cells as we now grow them is accompanied by a disappointing biochemical stability. In these cultures there is as yet no counterpart to the galaxy of auxotrophic mutants revealed in bacteria by the penicillin technique; and prototrophic mutants also are apparently more difficult to detect in animal cells than they are in bacteria or yeast. Do mutations occur less frequently in these cultures of somatic diploid human cells than they do in microorganisms, or do we merely lack the proper techniques for their selection?

III

Be that as it may, the microbiologist's stake in animal cells is obviously more than an interest in comparative biochemistry. The animal cell phagocytoses bacteria; and this interaction between the microorganism and the host cell is now being studied in a number of laboratories. It is an animal cell which produces antibodies to bacteria and viruses. The *in vitro* production of antibodies has been demonstrated in primary cultures of cells which had been appropriately conditioned *in vivo*; and ultimately it will probably be achieved in serially propagated cell cultures. Only a beginning has been made in the study of the interaction of animal cells and viruses; but the analysis of that interaction promises to be no less rewarding than that of bacteria and bacterial viruses. Finally, the recent convincing evidence in a number of laboratories that viruses are causally related to the production of a number of animal tumors opens yet another exciting vista for exploration by the microbiologist.

It is evident that in this, as in many other areas, the content of our science is changing, and changing rapidly. Even the name of our Society is an anachronism. It requires a highly elastic definition of bacteriology to encompass the content of this year's program; and it has been frequently suggested that we should change our name to, *e.g.*, the Society of American Microbiologists. As recently as 1940 the genus *Microbiologist* had not been recognized by the editors of Webster's dictionary. The term now has currency and respectability; but I suggest that it already requires re-evaluation. Strictly speaking, the microbiologist is a biologist who studies microbes; but I submit that we are more precisely characterized as micro-biologists, or perhaps better, cyto-biologists, biologists broadly concerned with the study of the cell. The structure and functions of the cell, and the mutual interrelations of animal cells, bacteria and viruses become ever more susceptible of experimental analysis. We should perhaps take formal recognition of the fact that this development is having a profound and obvious effect on our preoccupations as a Society and as individual scientists.